Bioefficacy of Essential Oil Based Nano-insecticidal Formulations against *Anopheles stephensi* and *Culex quinquefasciatus*

SYNOPSIS

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INTRODUCTION

Mosquitoes are the most diverse species of Arthropods which found in all the habitats. There are approximately over 3,500 species of mosquitoes grouped into 41 genera and comprise in the Family Culicidae, it is the deadliest family in the world **(Aina *et al.*, 2009)**. Mosquitoes are serious threat because they act as vectors of various diseases (malaria, dengue, yellow fever, chikungunya, filariasis etc.) that can be morbid and fatal to the human health **(Bhan *et al.*, 2015)**. Owing to lack of proper medication and vaccination for treating mosquito-borne diseases, an alternative, effective and more promising approach used to combat the mosquito population is at their egg, larval and pupal stages **(Sugumar *et al.*, 2014)**. The application of various adulticides and repellents can only reduce the adult population temporarily because many of them migrate from the treated areas. While larvicides kill all the larvae because during this stage, the mosquitoes are less motile, confined within their aquatic habitats and does not migrate **(Amer and Mehlhorn, 2006)**. In most of the species, the adult females lay their eggs in stagnant water; some lay eggs near water's edges; others attach their eggs to aquatic plants.

WHO has estimated 27,000 people died in Asia and 4,45,000 people died globally due to malaria in 2016 and approximately over 25 million people worldwide affected by lymphatic filariasis **(WHO, 2018)**. Many approaches have been tried to control mosquitoes but the control is mainly based on synthetic insecticides. Indiscriminate and repeated application of such insecticides to diminish the mosquitoes has created physiological resistance to these disease vectors. Moreover, they resulted in serious environmental issues such as environmental pollutions as well as toxicity to aquatic ecosystem and adversely harm the beneficial non-target organisms **(Castillo *et al.*, 2017)**. Potential risks due to the application of synthetic insecticides lead to an urgent need to procreate safe, natural, eco-friendly and efficient alternative to mosquito management, which is more specific and has little or no harmful effects **(Ghosh *et al.*, 2013; Rathy *et al.*, 2015).** Unlike chemical larvicides which are based on a single active ingredient, plant derived larvicides are the safer and attractive alternative because they comprises high amount of multiple active compounds which prevents to develop insect resistance when they use in their natural or crude form **(Malar *et al.*, 2017).**

Essential oils are concentrated hydrophobic liquid containing many volatile aroma compounds derived from the plants. The oil is "essential" in the sense that it contains the essence of the plant. Essential oils are extracted by the hydrodistillation of plant materials, have a long history of use in fragrance and flavouring in the perfume/cosmetic and food industries **(Ram *et al*., 2012).** But recently, essential oils have been suggested as an alternative source for insect control, because they constitute a rich source of bioactive compounds that are effective, biodegradable and easily applicable as mosquito larvicidal agents **(Jayaraman *et al*., 2015).** Phytochemicals present in essential oils are advantageous due to their eco-safety, target-specificity, non-development of resistance, reduced number of application and higher acceptability with low or no mammalian toxicity **(Benelli, 2016).** Technocrats have reported the essential oils are effective as insect repellent, antifeedent, insecticide, growth suppressor and reproduction inhibitor **(Mossa, 2016)**.

Nanotechnology is a multidisciplinary approach which involves creation and utilization of different systems on a nanometric scale. Several types of nanoformulations have been reported, including nanoemulsions, which are dispersed system constituted by two immiscible liquids and emulsifier **(Duarte *et al*., 2015).** The utilization of plant-borne biochemicals for preparation of nanoformulations is low-cost, single-step and eco-friendly method. The green synthesis of nanoformulations is beneficial over the other chemical and physical methods, since it is not necessary to use high pressure, high energy, high temperature or any toxic chemicals **(Gupta *et al*., 2016).** Nanoemulsions are thermodynamically stable; possess high bioavailability and optically transparent or translucent because the dispersions of oil and water stabilized by an interfacial film of surfactant molecules and one of the most efficient dispersed nanosystems of droplet size range upto 200nm **(Patel and Joshi, 2012).** Nanoemulsions prepared by essential oils, are one of the novel policies for control mosquitoes. The morphological and biochemical analysis are valuable in assessing and findings the toxicological impact of phytochemicals on the insects.

Hence, the present study is designed to evaluate the larvicidal efficacy of certain essential oils as well as their nanoinsecticidal formulations against *Anopheles stephensi* and *Culex quinquefasciatus*. Further, the impact of the most potent nanoformulation on the morphological and biochemical parameters of the treated target organisms will also be undertaken.

REVIEW OF LITERATURE

In the view of health and economic difficulties generated by increased mosquitoes population and resistance to synthetic insecticides, alternative methods have gained increasing attention. The use of nanomaterials in mosquito management is a developing field in the recent decade therefore; most of the workers have been paid their attention towards the use of nanoformulations in the field of vector control. The literature consulted is summarized as follows.

- **Essential Oils/Extracts in Mosquito Management :**

Amer and Mehlhorn (2006) assessed the larvicidal activity of essential oils obtained from the various plants including *Citrus limon* screened against *Aedes*, *Anopheles* and *Culex*. **Siddiqui *et al.* (2009)** studied the fractions and larvicidal activity of the flower essential oil of *Azadirachta indica* against *Anopheles stephensi*. **Dua *et al*. (2009)** evaluated the larvicidal properties of the emulsified neem oil formulation against *Anopheles stephensi*, *Culex quinquefasciatus* and *Aedes aegypti*. **Michaelakis *et al*. (2009)** elucidated the fruit essential oil of sweet orange (*Citrus sinensis*), lemon (*C. limon*) and bitter orange (*C. aurantium*) against *Culex pipiens*. **Sutthanont *et al*. (2010)** examined the chemical composition and larvicidal activity of essential oils of five edible plants including *Citrus reticulata* against the pyrethroid susceptible and resistant strains of *Aedes aegypti*. **Paiva *et al*. (2011**) determined the biological and larvicidal properties of some medically important plants and their compounds including *Argemon mexicana* against *Aedes aegypti*. **Rajkumar *et al*. (2011)** studied the larvicidal properties of leaf essential oil of *Coccinia indica* against the embryonic and larval stages of *Anopheles stephensi*. **Manimaran *et al*. (2012)** assessed the larvicidal and knockdown abilities of various essential oils derived from *Citrus sinensis* and many other plants screened against *Culex quinquefasciatus*, *Aedes aegypti* and *Anopheles stephensi*. **Warikoo *et al*. (2012)** investigated the larvicidal and repellent properties of hexane extract of the leaves of *Citrus sinensis* against the larvae and female adults of *Aedes aegypti*. **Palazzolo *et al*. (2013)** reported the potential of essential oils of different Citrus plants as insecticidal agents against *Culex pipiense*. **El-Hawary *et al*. (2013)** assessed the chemical composition, antioxidant, antibacterial, antifungal and larvicidal biological activities of essential oils obtained from leaf and flower of *Azadirachta indica* against *Culex pipiens*.

El-Akhal *et al.* (2015) determined the larvicidal bioefficacy of essential oils of two plants, *Citrus aurantium* and *Citrus sinensis* screened against *Anopheles labranchiae.* **Abou-Elnaga (2015)** determined the larvicidal activity of leaf and seed extracts of *Argemone mexicana* against *Culex pipiens* and *Aedes aegypti*. **Benelli (2015)** presented a review on the ovicidal activity of several essential oils and their fractions against three important mosquito vectors *Anopheles stephensi, Aedes aegypti* and *Culex quinquefasciatus*. **Jayakumar *et al*. (2016)** examined the larvicidal and pupicidal activities of essential oils collected from the different plants including *Citrus aurantium* against *Culex quinquefasciatus.* **Rao *et al.* (2016)** assessed the larvicidal and repellent activities of essential oil obtained from seeds of *Citrus limetta* against the dengue vector, *Aedes aegypti*. **Sanei-Dehkordi *et al.* (2016)** determined the chemical composition and comparative efficacy of peel essential oils of *Citrus aurantium* and *Citrus paradise* against the larvae of *Anopheles stephensi*. **Sivaraman *et al*. (2016)** reported larvicidal property of various extracts of *Argemone mexicana* seeds against *Culex quinquefasciatus* and *Aedes aegypti*. **Hikal *et al*. (2017)** summarized a review on biological control of insects by using botanical insecticides as a replacement to chemical or synthetic insecticides to control different pests and three species of mosquitoes. **Malar *et al*. (2017)** evaluated the bioefficacy of *Citrus grandis* peel essential oil against larvae of *Aedes aegypti* and its effect on non-target organisms. **Castillo *et al*. (2017)** investigated the pupicidal, adulticidal, repellent, and oviposition-deterrent activities of several plant-derived essential oils including *Citrus sinensis* on *Aedes aegypti.* **Badawy *et al*. (2017)** determined the chemical composition of three essential oils isolated from peels of *Citrus reticulate, Citrus sinensis* and their larvicidal and adulticidal activities against *Culex pipiens.* **Mohammed *et al*. (2017)** studied the toxicity induced by essential oil of *Coccinia grandis* leaves against the larvae of *Anopheles stephensi, Aedes aegypti* and *Culex quinquefasciatus.* **Jemberie *et al*. (2017)** evaluated the larvicidal properties of leaf and seed extracts of *Argemone mexicana* against *Culex quinquefasciatus*. **Dosoky and Setzer (2018)** reviewed a report on the antimicrobial, larvicidal activities along with safety considerations of essential oils collected from the different species of Citrus plants against *Anopheles stephensi, Culex quinquefasciatus* and *Aedes aegypti* mosquitoes.

- **Combined activity of Essential oils/Extracts in Mosquito Management**

Maheswaran and Ignacimuthu (2012) examined the joint effect of *Azadirachta indica* and *Pongamia glabra* as larvicidal, ovicidal and oviposition deterrent activities against *Aedes aegypti* and *Anopheles albopictus*. **Intirach *et al*. (2012)** evaluated the chemical composition and larvicidal activity of some essential oils and their combined action against *Anopheles cracens*. **Bansal *et al*. (2014)** studied the synergistic and antagonistic efficacy of *Solanum xanthocarpum* and *Withania somnifera* on *Anopheles, Aedes* and *Culex* larvae. **Noosidum *et al.* (2014)** determined the behavioural responses and repellent activities by using the combination of two essential oils and compared the effect of single and combination of essential oils on *Aedes aegypti*. **Bhan *et al*. (2015)** evaluated the synergistic properties of the combination of *Cuscuta reflexa* and temephos against the larvae of *Anopheles stephensi* and *Culex quinquefasciatus*. **Zibaee and Khorram (2015)** studied the synergistic and knockdown effect of *Eucalyptus globulus* and *Rosmarinus officinalis* essential oils against *Periplaneta americana, Culex pipiens, Anopheles stephensi* and *Musca domestica*. **Das *et al.* (2015)** investigated the individual and synergistic mosquito-repellent ability of essential oils obtained from *Curcuma longa, Pogostemon heyneanus* and *Zanthoxylum limonella* against *Aedes albopictus*. **Tyagi *et al*. (2016)** assessed repellent efficacy of the mixtures in different concentrations of some essential oils isolated from five plants against *Aedes albopictus.* **Rios *et al*. (2017)** analysed the larvicidal bioefficacy as well as joint activity of essential oils collected from certain aromatic plants on *Aedes aegypti.*

- **Nanoformulations in Mosquito Management**

Nuchuchua *et al.* (2009) evaluated the composition and repellent activity of nanoemulsions prepared by mixture of three essential oils; citronella, hairy basil (*Ocimum americanum*) and vetiver against *Aedes aegypti*. A**njali *et al*. (2012)** determined the formulation and larvicidal efficacy of nanoemulsion containing *Azadirachta indica* essential oil against *Culex quinquefasciatus.* **Ghosh *et al.* (2013)** assessed the preparation, characterization and larvicidal activity of nanoemulsion formulated by *Ocimum basilicum* (basil) against dengue vector *Aedes aegypti*. **Sugumar *et al*. (2014)** examined the larvicidal, antibacterial, histological and biochemical activities of *Eucalyptus globulus*

oil based nanoemulsion screened against *Culex quinquefasciatus*. **Duarte *et al*. (2015)** evaluated the potential of nanoemulsion prepared by using essential oil of *Rosmarinus officinalis* against the larvae of *Aedes aegypti*. **Benelli (2016)** studied plant mediated green biosynthesis of nanoparticles against medical and veterinary important mosquitoes *viz, Aedes aegypti, Anopheles stephensi* and *Culex quinquefasciatus*. **Ramar *et al.* (2017)** evaluated the knockdown and adulticidal efficacy of nanoemulsion containing essential oil of *Ocimum sanctum* (Tulsi) against *Aedes aegypti* and *Culex quinquefasciatus.* **Vino and Durairaj (2017)** investigated the larvicidal ability of *Azadirachta indica* and *Lantana camera* oils loaded nanoemulsions used against *Culex quinquefasciatus* and *Aedes aegypti*. **Benelli (2018)** described the mechanism of action and toxic effect of nanoparticles on medically important mosquitoes in a detailed review article. **Sogan *et al.* (2018)** assessed the preparation, characterization and larvicidal activity of nanoemulsion of castor oil (*Ricinus communis*) against the malaria vector, *Anopheles culicifacies.*

- **Morphological Studies**

Khater and Shalaby (2008) evaluated some morphological abnormalities in the larvae of *Culex pipens* after the exposure of fenugreek, earth almond, mustard and olibanum derived essential oils. **Kumar *et al*. (2011)** analyzed the morphological alterations in *Aedes aegypti* after treated with three species of peppers. **Saranya *et al*. (2013)** studied the morphological deformities induced by leaf extract of *Spathodea campanulata* against larvae and pupae of *Aedes aegypti*. **Pratti *et al*. (2015)** assessed the characterization of structural damage induced by the essential oil of *Schinus terebinthifolia* (Brazilian pepper) on *Stegomyia aegypti*, a dengue vector. **Velu *et al*., (2015)** studied some morphological changes caused by peel extracts of *Arachic hypogaea* in the larvae of *Aedes aegypti*. **Yu *et al*. (2015)** evaluated the morphological changes in the larvae of *Aedes aegypti* due to the phytochemical of three seaweeds extracts. **Soonwera and Phasomkusolsil (2016)** examined the impact of *Cymbopogon citratus* (Lemongrass) and *Syzyium aromaticum* (Clove) essential oils on the morphology of *Aedes aegypti* and *Anopheles dirus* larvae. **Chantawee and Soonwera (2018)** investigated the morphological aberrations induced by essential oil of *Alpinia galanga, Anethum graveolens, Foeniculum vulgare* and *Pimpinella anisum* plants against *Aedes aegypti.*

- **Biochemical Studies**

Vontas *et al*. (2000) assessed the Glutathione S-transferase (GST) activity performed by spectrophotometric measurements against *Anopheles gambiae*. **Hunt *et al*. (2005)** investigated the monooxygenase and glutathione S-transferase activity for characterization of pyrethroid resistance against *Anopheles funestus*. **Pethuan *et al.* (2007)** studied the mechanism for insecticide resistance in *Aedes aegypti* and *Aedes albopictus* by their biochemical analysis. **Morou *et al*. (2010)** investigated colorimetric assay for determination of Glutathione-s transferase activity associated with DDT resistance in mosquitoes such as *Aedes aegypti*. **Akiner and Ekşi (2015)** investigated the insecticide resistance and biochemical mechanisms of *Culex pipiens* by Glutathione S-transferase (GST) and Non-specific esterases (NSEs) enzyme analysis. **Dahan-Moss and Koekemoer (2016)** analysed the activities of numerous enzymes of esterases family in adults of malaria vector, *Anopheles funestus.* **Fouad *et al*. (2017)** examined the effect of *Cassia fistula* and its nanoparticles on the biochemical parameter of *Aedes albopictus* and *Culex pipiens.* **Ga'al *et al*. (2018)** studied the impact of silver nanoparticles on total proteins, acetylcholine esterase and phosphatase enzymes in the larvae and pupae of *Aedes albopictus*. **Mishra *et al.* (2018)** investigated the biochemical parameters such as glutathione S-transferase and alkaline/acid phosphatase altered by neem-laced urea nanoemulsion against *Aedes aegypti* and *Culex tritaeniorhynchus.*

In the light of above described literature, the selected plants are proved effective as insecticide and play a vital role in controlling the mosquitoes. Many of the above mentioned researchers have been studied the various biological properties of selected plant materials but very less attention has been paid to their nanoformulations in the mosquito management. So based on these premises, this study has been undertaken to assess and compare the larvicidal efficacy of plant derived essential oils and their nanoformulations against *Anopheles stephensi* and *Culex quinquefasciatus* along with morphological and biochemical analysis to unravel the possible mode of action which leading to the mortality of mosquito larvae. The pilot work has been done on the selected plant materials in the laboratory.

OBJECTIVES

The main purpose of the present study is to control mosquito borne diseases through the management of the related vector mosquito at their larval stage. The following objectives will be selected for the present study:

- Larvicidal bioefficacy of *Argemone mexicana, Azadirachta indica, Citrus limetta, C. limon* and *C. reticulata* essential oils against *Anopheles stephensi* and *Culex quinquefasciatus.*
- Combined larvicidal activity of two most effective essential oils against *An. stephensi* and *Cx. quinquefasciatus.*
- Formulation, characterization and bioefficacy of the most effective combination based nanoinsecticidal formulations against *An. stephensi* and *Cx. quinquefasciatus*.
- Impact analysis of the most effective nanoformulation on the morphological and biochemical parameters of treated target organisms.

MATERIALS AND METHODS

Target Organisms

The larvae of *Anopheles stephensi* and *Culex quinquefasciatus* are selected for the present study.

- ***Anopheles stephensi***

It is a primary mosquito vector of malaria in urban India and bite mainly during dusk and dawn. About 12% of malaria cases in India are due to *Anopheles stephensi*. The development of protozoa, *Plasmodium* in *Anopheles* begins with the ingestion by female adult mosquito of blood containing gametocytes during blood feed via infected human.

- ***Culex quinquefasciatus***

It is commonly known as southern house mosquito and transmits zoonotic diseases that affect human as well as animals, such as lymphatic filariasis or elephantiasis. It is most common vector in urban and semi-urban areas of Asiatic regions. It breeds profusely in dirty water collections including stagnant drains, cesspools, septic tanks, burrow pits and almost all organic polluted water bodies.

Plants Selected

1) ***Argemone mexicana*** **[Family: Papaveraceae, Common name: Pricky poppy]**

It is native of tropical America and in India, it grows in the temperate region and it has tolerance of drought and poor soil. It has bright yellow coloured latex and though poisonous to grazing animals, that's why rarely eaten. It is wasteland weed present everywhere; 1-3 feet in height and the leaves are deeply lobed and thorny margins with white venation **(Priya and Rao, 2012)**. The whole plant and various parts of the plant are used as medicine to treat many diseases. The seeds are resemble to the seeds of mustard and contains 22-36% pale yellow, non-edible oil called Argemone oil. Larvicidal properties of extracts of leaves and seeds of *Argemone mexicana* against *Culex quinquefasciatus* and *Aedes aegypti* have been reported by **Abou-Elnaga, 2015**; **Sivaraman *et al*., 2016** and **Jemberie *et al*., 2017,** therefore previous studies show that it has potential to control mosquitoes.

2) *Azadirachta indica* [Family: Meliaceae, Common name: Neem]

It is also known as Indian Iliac and native to Indian subcontinents i.e., India, Nepal, Pakistan, Bangladesh and Sri Lanka. Neem is a fast growing evergreen tree that can reach upto height 15-20 m. The white and fragrant flowers are arranged in drooping axillary panicles which are 25 cm long. The inflorescences bear from 150 to 250 flowers and these are proandrous and bisexual. Neem flowers are used to treat nausea, intestinal worms and also in aromatherapy because of their calming effect. Essential oils of leaves and flowers of *Azadirachta indica* against some stored grain pests and *Culex pipiens* larvae have been studied by **Ram *et al*., 2012** and **El-hawary *et al*., 2013** respectively**.** The previous investigations revealed that the different parts of neem plant possess anti-mosquito activities.

3) *Citrus limetta* [Family: Rutaceae, Common name: Sweet lime]

It is native to southern regions of Iran and known as sweet lime because it is close relative of the lemon. It is one of the most important citrus plants which used in the world. It is a small tree upto 8 m in height with irregular branches. Essential oil occurs naturally in special oil glands, which present in flowers, leaves and peels **(Palazzolo *et al*., 2013).** The leaves are compound with acuminate leaflet 5 to17cm long and 3-8 cm wide. Fruits and leaves are used to treat the common cold, fever, decreasing cholesterol level, regulating blood pressure and inflammation. The larvicidal bioassay of seed essential oil of *Citrus limetta* on *Aedes aegypti* has been investigated by **Kumar, 2012** and **Rao *et al*., 2016.** Many studies have been focused on the application of *Citrus limetta* oil to combat mosquitoes.

4) *Citrus limon* [Family: Rutaceae, Common name: Lemon]

The lemon is a small evergreen flowering plant which is native to South Asia and North eastern India. The tree's ellipsoidal yellow fruit is used for culinary and non-culinary purposes and cultivated in many countries all over the world. Lemon juice contains slightly more citric acid than lime juice **(Vekiari *et al*., 2002).** Although juice is the main commercial product of lemons but essential oils are considerably important as economically, scientifically and used as active trade in many industries. Larvicidal effect of *Citrus limon* oil (peel) against *Anopheles stephensi, Aedes aegypti* and *Culex quinquefasciatus* examined by **Amer and Mehlhorn, 2006** and **Manimaran *et al*., 2012.** Many researchers have been reported the effectiveness of lemon plant products as efficient to mosquitocide.

5) *Citrus reticulata* [Family: Rutaceae, Common name: Kinnow]

Rutaceae family comprises of about 140 genera and 1,300 species. They are smaller and oblate, rather than spherical like the common oranges **(Praveen *et al*., 2014).** The taste is considered less sour as well as sweeter and stronger. Tree is moderate sized 3-4 m in height, vigorous; generally have thorns and a tree can yields approximately 5-7 thousand fruits. The leaves are shiny and green, rather small. Petioles are short; almost wingless or slightly winged. Citrus plants have potential commercial value because of its attractive and pleasant aroma. The larvicidal efficacy of *Citrus reticulata* essential oil of peels has been evaluated against *Culex pipiens* previously by **Badawy *et al*., 2017.**

Methods

- **Collection of Plant materials and Isolation of Essential Oils :**

Aerial part of *Argemone mexicana,* flowers of *Azadirachta indica,* leaves of *Citrus limetta, C. limon* and *C. reticulata* will be collected from the various places of Agra. The collected plant materials will be washed with tap water to remove all the unwanted impurities. In order to carry out the analysis, plant materials and water will be subjected to hydrodistillation for 6 hours by Clevenger apparatus. After manual collection of the essential oil, traces of remaining moisture present in the oil will be removed over anhydrous sodium sulphate (**Mohammed *et al*., 2017).** The isolated oil will be stored under refrigeration for further use. Yield of oils will be calculated from the weight of fresh plant material of related plant.

- **Rearing of Target Organisms :**

The efficacy will be performed against larvae of *Anopheles stephensi* and *Culex quinquefasciatus* mosquitoes. The immature mosquito's larvae will be collected from the nearby natural oviposition sites. The larvae will be maintained at 27± 2°C with 75±5% relative humidity and 14:10 hours day/night cycle in dechlorinated water. Larvae will feed with powdered yeast and dog biscuits in 2:1 ratio. Pupae will be transferred from the trays to a cup containing tap water and then placed into screened cages (23×23×32 cm) diameter. The adults will be reared in the glass cages of 30×30×30 cm dimension. The adult's colony will be provided with 10% sucrose solution with periodically blood feed on shaving rats. After 3 days, ovitrap will be kept inside the cages and the eggs will be collected

and transfer to the enamel tray at the same conditions. The third instars larvae will be used further in the bioassay **(Sogan *et al*., 2018).**

- **Larvicidal Bioefficacy :**

Essential oils of selected plant materials will be diluted in ethanol to obtain stock solutions of desired strength. Different test concentrations will be prepared in tap water from these stock solutions for the exposure of target mosquito larvae. The larval bioassays will be performed according to the standard WHO protocol **(WHO, 2005)**. Twenty laboratory acclimatized larvae (third instar) of each species will be exposed independently in a beaker containing 199 ml water and 1ml of desired concentration of stock solution. Minimum three replicates will be kept for each concentration along with the control.

- **Combined Bioefficacy :**

Joint bioefficacy of two most effective essential oils will be carried out in different combinations against the larvae of *Anopheles stephensi* and *Culex quinquefasciatus* as per above WHO protocol. **(Bansal *et al*., 2014)**

- **Preparation of Nanoinsecticide/Nanoemulsion Formulation :**

The nanoemulsion formulation will be prepared by using the most efficient combination of two essential oils with different ingredients such as Tween 20 (surfactant), glycerine/ethanol/ethylene glycol (co-surfactant) and distilled water as per the requirements. The essential oils, surfactant and co-surfactant will be stirred at 800 rpm by using magnetic stirrer for 30 minutes and then the water added in drop wise manner. The mixture will be stirred at 800 rpm for 60 minutes **(Duarte *et al*., 2015)** and then the mixture will be sonicated for 1-2 hours by using high energy sonicator. The nanoemulsion will be stored in refrigerator for their characterization and bioefficacy evaluation **(Anjali *et al*., 2012).**

- **Nanoinsecticide/Nanoemulsion Bioefficacy :**

Above prepared nanoinsecticide or nanoemulsion formulations will be used for their bioassay against the target organisms as per above standard WHO protocol **(WHO, 2005).**

- **Statistical analysis :**

The larval mortality will be observed after 24 and 48 hours of the exposure period. The mortality data will be subjected to determine the lethal concentrations (LC_{50}) and (LC_{90}) by probit analysis **(Finney, 1971)**. When the control mortality ranged from 5-20%, the observed mortality percentage will be corrected by using Abbott's formula **(Abbott, 1925).** But when the bioassay will be showed more than 20% larval mortality in control, the experiment will be discarded and repeated.

$$\text{Abbott's formula} = \frac{\text{Percentage of test mortality} - \text{Percentage of control mortality}}{100 - \text{Percentage of control mortality}} \times 100$$

- **Morphological Analysis :**

Treated larvae will be studied for morphological alterations under the light microscope. For the observation, larvae would be mounted on the microscopic slide with Hoyer's medium. Morphological changes if any in body segments including head, abdomen, thorax and anal gills will be observed and compared with the control **(Kumar *et al*., 2011)**.

- **Biochemical Analysis :**

Biochemical and physiological alterations will be evaluated by biochemical analysis of treated target mosquito larvae by using the procedures described by **WHO, 1998**. Control and treated larvae of selected target organisms will be washed with distilled water and dried with tissue paper to remove the adhering water to their body surfaces. The larvae will be homogenized in Eppendorf TM tubes using a hand homogenizer in 1ml of phosphate buffer (pH 7.0) for determination of acetylcholinesterase, alkaline phosphatase and glutathione S-transferase analysis **(Fouad *et al*., 2017)**. The whole body homogenates will be centrifuged at 10,000 rpm for 15 minutes and the clear supernatants will be used for biochemical analysis.

- **Estimation of Acetylcholinesterase:**

Acetylcholinesterase activity in the control and test larval homogenate will be determined by **Wierenga and Hollingworth, 1992** method. Each homogenate will be mixed with sodium phosphate buffer (pH 7.5). Then 5,5-dithio-bis-(2-nitrobenzoate) and acetylthiocholine iodide will be added for a total volume of 1ml. After incubation for 5 minutes at room temperature, the reaction will be measured at 400 nm using UV spectrophotometer against suitable reagent blank **(Foaud *et al*., 2017)**.

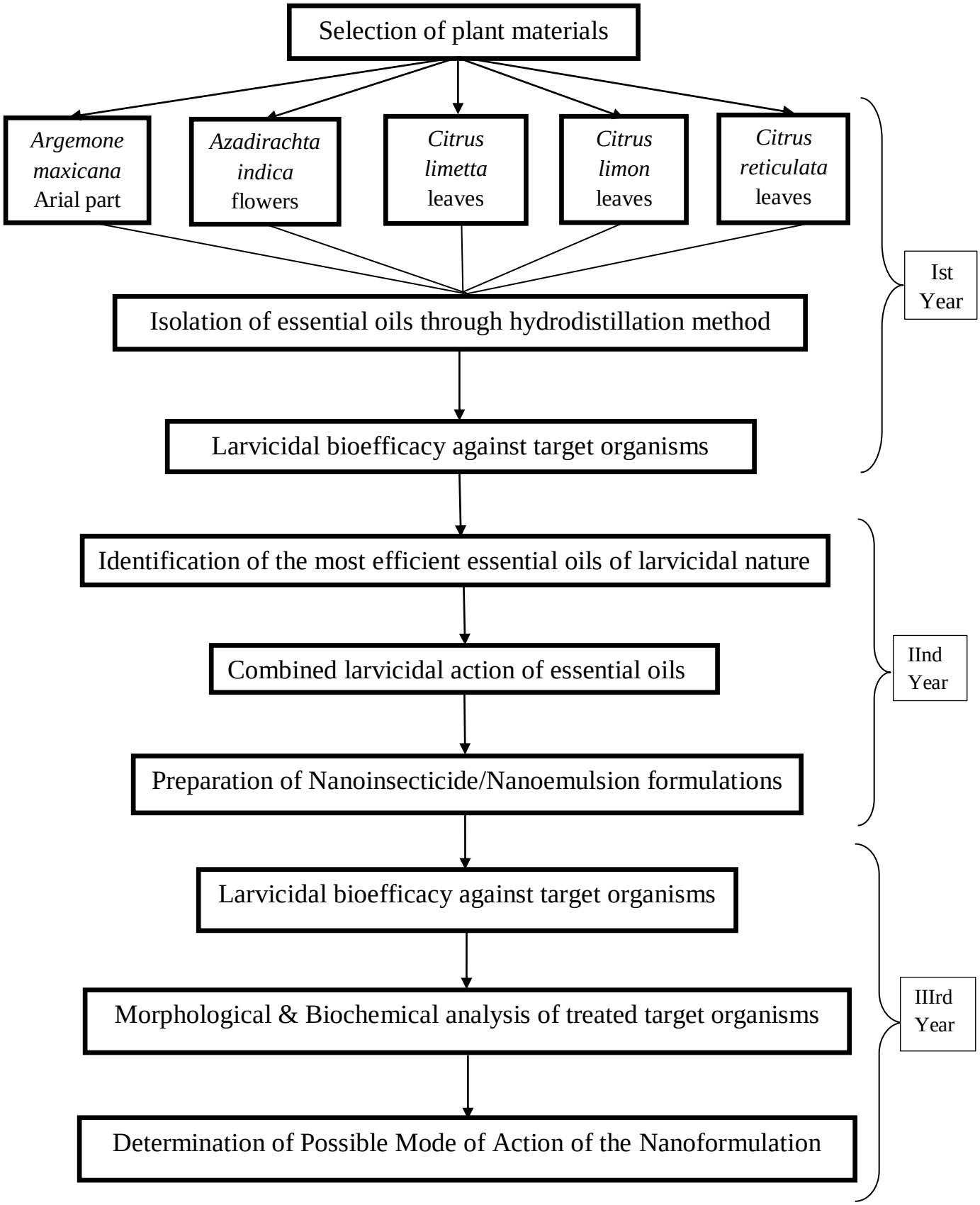
- **Estimation of Alkaline Phosphatase:**

The levels of alkaline phosphatases (ALP) in the larval homogenates will be measured by the method of **Asakura, 1978**. The homogenate will be mixed with Tris-HCl buffer (pH 8.0) and p-nitrophenyl phosphate. After incubation for 15 minutes at 37°C in water bath, the enzymatic reaction will be stopped by adding NaOH solution and centrifuged at 4000 rpm for 5 minutes. The absorbency of the resulting clear supernatant from each sample will be read at 440 nm against the appropriate reagent blank **(Ga'al *et al*., 2018)**.

- **Estimation of Glutathione S-transferase:**

Standard Glutathione S-transferase spectrophotometric assays will be performed by **Habig *et al*., 1974** method through monitoring the formation of the conjugate of 1, 2-dichloro-4-nitrobenzene (DCNB) and reduced glutathione (GSH). The iodide-releasing reaction will be carried out and optimised with GSH and iodoethane in phosphate buffer (pH 8.2) and enzyme source in a total volume of 100 ml at 25°C. The reaction will be incubated for 30 minutes at 37°C. Blue colour will be developed immediately after addition of 50 ml starch solution and 100 ml acidified peroxide solution. The blue colour will be measured at 610 nm by using spectrophotometer **(Morou *et al*. 2010)**.

METHOHOLOGY AT A GLANCE


Selection of plant materials
Argemone maxicana Arial part
Azadirachta indica flowers
Citrus limetta leaves
Citrus limon leaves
Citrus reticulata leaves
Isolation of essential oils through hydrodistillation method
Larvicidal bioefficacy against target organisms
Ist Year
Identification of the most efficient essential oils of larvicidal nature
Combined larvicidal action of essential oils
Preparation of Nanoinsecticide/Nanoemulsion formulations
IInd Year
Larvicidal bioefficacy against target organisms
Morphological & Biochemical analysis of treated target organisms
Determination of Possible Mode of Action of the Nanoformulation
IIIrd Year

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